Effective glycaemic control critically determines insulin cardioprotection against ischaemia/reperfusion injury in anaesthetized dogs

Qiujun Yu1†, Ning Zhou2†, Ying Nan3†, Lihua Zhang4, Yan Li1, Xiaoke Hao5, Lize Xiong6, Wayne Bond Lau7, Xin L. Ma7, Haichang Wang1*, and Feng Gao1,3*

1Department of Cardiology, Xijing Hospital, Fourth Military Medical University, Xi’an, China; 2Department of Cardiology, Hospital of Shaanxi Provincial Corps of Chinese People’s Armed Police Forces, Xi’an, China; 3Department of Cardiology, Hospital of Shaanxi Provincial Corps of Chinese People’s Armed Police Forces, Xi’an, China; 4Department of Physiology, Fourth Military Medical University, Xi’an, China; 5Department of Geriatrics, Tangdu Hospital, Fourth Military Medical University, Xi’an, China; 6Department of Anesthesiology, Xijing Hospital, Fourth Military Medical University, Xi’an, China; and 7Department of Emergency Medicine, Thomas Jefferson University, Philadelphia, PA, USA

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Aims
Experimental evidence has shown significant cardioprotective effects of insulin, whereas clinical trials produced mixed results without valid explanations. This study was designed to examine the effect of hyperglycaemia on insulin cardioprotective action in a preclinical large animal model of myocardial ischaemia/reperfusion (MI/R).

Methods and results
Anaesthetized dogs were subjected to MI/R (30 min/4 h) and randomized to normal plasma insulin/euglycaemia (NI/NG), normal-insulin/hyperglycaemia (NI/HG), high-insulin/euglycaemia (HI/NG), and high-insulin/hyperglycaemia (HI/HG) achieved by controlled glucose/insulin infusion. Endogenous insulin production was abolished by peripancreatic vessel ligation. Compared with the control animals (NI/NG), hyperglycaemia (NI/HG) significantly aggravated MI/R injury. Insulin elevation at clamped euglycaemia (HI/NG) protected against MI/R injury as evidenced by reduced infarct size, decreased necrosis and apoptosis, and alleviated inflammatory and oxidative stress (leucocyte infiltration, myeloperoxidase, and malondialdehyde levels). However, these cardioprotective effects of insulin were markedly blunted in hyperglycaemic animals (HI/HG).

In vitro mechanistic study in neonatal rat cardiomyocytes revealed that insulin-stimulated tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and Akt was significantly attenuated by high glucose, accompanied by markedly increased IRS-1 O-GlcNAc glycosylation following hypoxia/reoxygenation. Inhibition of hexosamine biosynthesis with 6-diazo-5-oxonorleucine abrogated high glucose-induced O-GlcNAc modification and inactivation of IRS-1/Akt as well as cell injury.

Conclusions
Our results, derived from a canine model of MI/R, demonstrate that hyperglycaemia blunts insulin protection against MI/R injury via hyperglycaemia-induced glycosylation and subsequent inactivation of insulin-signalling proteins. Our findings suggest that prevention of hyperglycaemia is critical for achieving maximal insulin cardioprotection for the ischaemic/reperfused hearts.

Keywords
Insulin • Hyperglycaemia • Myocardial ischaemia/reperfusion • Cardioprotection • Dog

1. Introduction
Accumulating evidence has documented the cardioprotective action of glucose–insulin–potassium (GIK) cocktail for the ischaemic hearts. However, GIK has not been uniformly effective in clinical practice as an adjunct to the contemporary management of acute myocardial infarction (AMI), and the reasons for the divergent results from basic studies and different clinical trials remain unclear. It is noteworthy that the beneficial effects of GIK in ischaemic heart, either in small animal models1–3 or in clinical trials,4–6 were reported with varying plasma glucose levels. Although it has been proposed that GIK or insulin administration without rigorous glycaemic control may not improve outcomes...
in AMI, it remains unevi-enced whether insulin-titrated euglycaemia maintenance is a pre-requisite for the cardioprotective effects of GIK.

Recent studies from our laboratory as well as others’ have demonstrated that insulin is the key component of GIK in cardioprotection and it protects ischaemic heart against apoptotic cell death and improves myocardial survival via activating cell-survival signalling. The main signalling pathway initiated by the insulin receptor for promoting cell survival originates with tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), leading to sequential activation of PI 3-kinase and its downstream effectors, including the Ser/Thr kinase Akt. Latest reports showed that many of the adverse effects of hyperglycaemia can be attributed to the increased routing of glucose through the hexosamine biosynthesis pathway (HBP). UDP-N-acetylglucosamine (UDP-GlcNAc), the final product of this pathway, is used by O-linked GlcNAc transferase as substrate for O-glycosylation of proteins. The sites of O-GlcNAc modification are often identical or adjacent to those of known phosphorylation; thus, O-GlcNAc modification may affect the phosphorylation state of important signalling proteins by competing with activating kinases. We hypothesized that hyperglycaemia-induced HBP overproduction may enhance O-GlcNAc modification of insulin-signalling proteins and thus affect GIK cardioprotection.

Therefore, the present study was designed to determine whether glycaemic control is a requisite to ensure insulin cardioprotective effect in myocardial ischaemia/reperfusion (MI/R) in a clinically relevant large animal model, and to investigate the molecular mechanisms by which hyperglycaemia interacts with insulin signalling.

2. Methods

2.1 Surgical preparation of animals

Animal experiments were performed in adherence with the US National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the Fourth Military Medical University Committee on Animal Care. Adult male mongrel dogs (10–14 months of age), weighing 14–18 kg, were fasted overnight, anesthetized with pentobarbital sodium (30 mg/kg, IV), intubated, and artificially ventilated with a positive pressure animal respirator. Temperature was maintained with a heating blanket, and anaesthesia was maintained with the absence of withdrawal response to paw pinch. Oxygen and 5% sodium bicarbonate were supplied when required to maintain arterial PaO2, PaCO2, and pH within physiological ranges (80–140 mmHg, 35–45 mmHg, and 7.35–7.45, respectively). Cannulation of the right femoral artery and vein provided means for aortic blood pressure measurement and supplemental anaesthetic/sodium bicarbonate administration. Cannulation of the left femoral artery and vein was prepared for blood sampling and drug infusion.

2.2 Pancreatic surgery and MI/R induction

To block endogenous pancreatic insulin release in response to fluctuating blood glucose, all peripancreatic vessels, including the anterior/posterior pancreaticoduodenal arteries and the dorsal, inferior, and caudate pancreatic arteries with accompanying veins, were clamped and ligated. The pancreatic head was ligated at its end proximal to the duodenum to block exocrine secretion through the pancreatic duct. MI/R (30 min/4 h) was introduced, as previously described with modest modifications.

2.3 Experimental protocols

As shown in Supplementary material online, Figure S1, dogs were randomized into four groups (n = 8/group): normal-insulin/euglycaemia group (NI/NG), normal-insulin/hyperglycaemia group (NI/HG), high-insulin/euglycaemia group (HI/NG), and high-insulin/hyperglycaemia group (HI/HG). The target plasma glucose level was set at 70–110 mg/dL (3.9–6.1 mmol/L) for the normoglycaemic groups, and 180–300 mg/dL (10.0–16.7 mmol/L) for the hyperglycaemic groups, according to previous reports from experimental and clinical studies. Fifty percentage of glucose was infused at a variable rate via a syringe pump to achieve and maintain targeted glucose levels. The target plasma insulin level was predetermined as 10–40 mU/L in the normal-insulin groups and 100–200 mU/L in the high-insulin groups. Pancreatic surgery (described above) was performed to block endogenous insulin release in response to exogenous glucose infusion. To achieve the targeted high-insulin level, GIK cocktail (glucose: 250 g/L; insulin: 60 U/L, Novo Nordisk, Denmark; potassium: 80 mmol/L) was administered intravenously via a syringe pump at the rate of 2 mL kg\(^{-1}\) h\(^{-1}\), initiated 5 min prior to and throughout reperfusion. Normal saline was given IV to sustain normovolaemia, monitored by arterial blood pressure (100–130/60–90 mmHg) and urine output (1–2 mL kg\(^{-1}\) h\(^{-1}\)). Intravenous lidocaine hydrochloride and low-energy direct current cardioversion were administered for preventing ventricular arrhythmia and fibrillation. No inotropic agents were used. Animals were sacrificed 4 h after reperfusion with an overdose injection of pentobarbital, and hearts were obtained for further experiments.

2.4 Biochemical assays

Blood glucose was measured by a clinical blood analyser (Lifescan, USA) at baseline, 15 min before, and 30 min after ischaemia, and every 10 min during the 4 h reperfusion period to allow aggressive and fine adjustment of glucose infusion. Plasma insulin and C-peptide concentrations were analysed in duplicate within a single assay using commercial radioimmunoassay kits (lower detection limit 2 mU/L and 0.15 μg/L, respectively, intra-assay variation <10%, Beijing North Institute of Biological Technology, China). Plasma levels of cardiac troponin-T (cTnT) were determined in duplicate by the Access Immunoassay System (Beckman Counter, Inc. Fullerton, USA) in a blinded manner.

2.5 Assessment of cardiac function

Left ventricular (LV) pressure was continuously monitored by a haemodynamic analysing system with a catheter inserted into the left ventricle via the right carotid artery. Heart rate (HR), LV systolic pressure (LVSP), and positive and negative maximum values of left ventricle dP/dt\(_{\text{max}}\) (± LVdP/dt\(_{\text{max}}\)) were derived by computer algorithms (Chengdu Instrument Co., China). Rate–pressure product (RPP), the product of HR and LVSP, was used as an index for ventricular performance.

2.6 Measurement of myocardial infarct size and apoptosis

Infarct size was measured using the Evans blue/2,3,5-triphenyltetrazolium chloride (TTC) double-staining method as described previously, and expressed as a percentage of infarct area (INF) over total area at risk (AAR) by weight. Immunohistochemical detection of caspase-3 activation and TUNEL assay for quantitative analysis of cell apoptosis were performed as described previously. Apoptotic index was expressed as the number of positively stained cells/total cells \(\times 100\%\).

2.7 Evaluation of tissue inflammation and oxidative stress

Leucocyte infiltration in perinecrotic myocardium was assessed in haematoxylin–eosin (HE)-stained paraffin sections. Tissue activity of myeloperoxidase (MPO), an enzyme exclusively existing in neutrophils, was determined as a quantitative index for leucocyte recruitment. One unit of MPO activity was defined as the quantity of enzyme hydrolyzing 1 mmol of p-nitrophenol per min at 25°C. Results were expressed as unit/mg protein. The content of malondialdehyde (MDA) and activity of superoxide dismutase (SOD) of the reperfused cardiac tissue were measured using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China) with a spectrophotometer (Beckman DU 640, USA) in a blinded manner. The sediment was...
resuspended and used for the Bio-Rad Protein Assay (Bio-Rad Laboratories) verifying tissue protein content.

2.8 Neonatal rat cardiomyocyte culture and simulated I/R

Sprague-Dawley rats of 1–2 days old were sacrificed by decapitation and isolation, and primary cultures of neonatal rat cardiomyocytes (NRCMs) were performed as previously described.18 NRCMs after 24 h culture were then randomized into six groups (see Supplementary material online, Figure S1): control group (Control) that was cultured in normal medium; Mannitol group (Mannitol) that was cultured in medium supplemented with 19.5 mM Mannitol; Normal glucose group (NG) that was cultured in medium containing 5.5 mM glucose; High-glucose group (HG) that was cultured in medium containing 25 mM glucose; Normal glucose plus insulin group (NG + Ins) that was cultured in medium containing 5.5 mM glucose supplemented with $10^{-7}$ M insulin; High-glucose plus insulin group (HG + Ins) that was cultured in medium containing 25 mM glucose supplemented with $10^{-7}$ M insulin. The last five groups received the respective culture for 48 h followed by simulated I/R (SI/R, 4 h/2 h) as described previously.18 Additional experiments were performed using NRCMs pre-incubated with 40 $\mu$M of 6-diazo-5-oxonorleucine (DON, an inhibitor of glutamine : fructose-6-phosphate amidotransferase) for 24 h before any additional reagents.

2.9 Determination of cell viability, LDH release, and cell apoptosis

Cardiomyocyte viability was determined by the staining of trypan blue, which is a vital dye excluded by viable cells with intact cell membranes and is well documented as an indicator of oncotic cell death. The NRCMs and culture supernatant after H/R were separately collected. LDH activities were measured by colorimetric monitoring of reaction kinetics according to the manufacturer’s instructions. The total LDH activity was obtained from adding LDH activities in the culture supernatant and the cell lysate together. The LDH release rate was expressed as supernatant LDH activities/total LDH activity $\times 100\%$. NRCM apoptosis was performed using Annexin V-fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI) double staining followed by flow cytometry.

2.10 Immunoprecipitation and western blot analysis

Proteins were extracted and immunoprecipitated with anti-IRS-1 antibody (rabbit polyclonal, Abcam) and 20 $\mu$L of Protein A/G agarose (Pierce) overnight at 4°C. The immunoprecipitation complexes were pelleted by centrifugation, washed, and resuspended in sample buffer, then boiled and analysed by SDS/10% PAGE with western blotting. IRS-1 proteins and total proteins electrophoresed on SDS–PAGE gels were transferred onto PVDF (polycytrnilydendifluoride, Millipore). The immunoblots were probed with GlcNAc (Abcam), IRS-1, phosphor-IRS-1 (Abcam), Akt, and phospho-Akt (Cell Signaling, Beverly, MA, USA) overnight at 4°C, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h. Blots were detected via enhanced chemiluminescence (Millipore).

2.11 Statistical analysis

Strict criteria were established to guarantee that all animals included in the final data analysis were healthy and exposed to comparable degrees of regional myocardial ischaemia. In total, four animals were excluded and subsequently replaced due to one of the following conditions: (i) post-mortem examination revealed the presence of heart worm infection, (ii) AAR/LV proportion by weight beyond 10–20% range, (iii) greater than three episodes of reversible ventricular fibrillation, or (iv) blood

Figure 1 Independently manipulated blood glucose and plasma insulin levels in canine MI/R models. Blood glucose (A), plasma insulin (B), and C-peptide (C) concentrations achieved and maintained at the preset target levels in corresponding groups during MI/R by controlled glucose/insulin infusion and peri-pancreatic vessel ligation. $n = 8$ per group, means $\pm$ SEM. $**P < 0.01$ vs. NI/NG. NI/NG: normal-insulin/euglycaemia group; NI/HG: normal-insulin/hyperglycaemia group; Hi/NG: high-insulin/euglycaemia group; Hi/HG: high-insulin/hyperglycaemia group.
glucose <50 mg/dL (2.8 mmol/L) or >350 mg/dL (19.4 mmol/L) at any time point during the experiment. All values are expressed as mean ± SEM. Results were analysed by one- or two-way ANOVA followed by Bonferroni’s post hoc test to further assess significance of differences among groups. Probabilities of <0.05 were considered statistically significant. All statistical tests were performed with GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1 Physical characteristics and metabolic profile

Animals from randomized groups showed no statistical differences in age and body weight, and baseline metabolic profiles were comparable among groups regarding to blood glucose and plasma insulin (see Supplementary material online, Table S1). Plasma insulin was maintained throughout the 4 h reperfusion period at a normal (20.9–44.4 mU/L) or elevated (124.6–176.0 mU/L) level (Figure 1B), independent of the two targeted blood glucose concentrations (3.7–6.9 mmol/L and 10.9–16.7 mmol/L) (Figure 1A). Plasma C-peptide, an indirect index of endogenous insulin secretion as the by-product of proinsulin splitting into insulin, maintained at the baseline level (0.9 ± 0.07 μg/L) throughout the experiment (Figure 1C), providing further evidence of successful pancreatic ligation. HE staining of the paraffin slides from ischaemic pancreas showed regional oedema, degeneration, and local autolysis (see Supplementary material online, Figure S2A and B). Plasma levels of pancreatic amylase, cTnT, and creatine kinase-MB (see Supplementary material online, Figure S2C and D), as well as echocardiographic haemodynamic indices (data not shown) showed no statistically significant differences between pancreatic-ligated vs. non-operated dogs subjected to MI/R, suggesting negligible systemic and cardiac effects of pancreatic surgery.

3.2 Myocardial infarction and contractile function following MI/R

Myocardial ischaemic area (AAR/LV) was comparable among all groups (Figure 2B). Hyperglycaemia alone (NI/HG) aggravated infarction as evidenced by enlarged areas of TTC-negatively stained myocardium (27.5 ± 1.3% in NI/HG vs. 17.2 ± 1.1% in NI/NG group, n = 6, P < 0.05) (Figure 2A). In contrast, exogenous insulin markedly reduced infarct size when blood glucose levels were clamped at constant normal levels (11.9 ± 1.0% in HI/NG vs. NI/NG, P < 0.05). Most importantly, insulin elevation failed to reduce infarct size in the setting of hyperglycaemia (HI/HG vs. NI/HG, P > 0.05). The plasma level of

![Figure 2](https://example.com/figure2.png)

**Figure 2** Myocardial infarction, necrotic injury, and contractile function following MI/R. Myocardial INF (A) and ischaemic area (AAR) (B), as well as plasma level of cTnT (C) were analysed at the end of 4 h reperfusion. Infarct size was expressed as the percentage of INF over AAR by weight. HR (D), RPP (E), and ±LVdP/dt max (F and G) were derived from LV pressure monitored throughout the experiment. n = 8 per group, means ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001.
cTnT, a recognized biomarker for myocardial necrotic injury, exhibited a similar trend as TTC staining (Figure 2C).

No significant differences in haemodynamic parameters were observed among all groups at baseline, before, or during myocardial ischaemia. NI/HG dogs showed a slight decline in HR (Figure 2D) and a significant decrease in RPP and LVdp/dt\text{max} during reperfusion (Figure 2E–G). Insulin infusion significantly enhanced RPP and LVdp/dt\text{max} at clamped euglycaemia, but not in the presence of hyperglycaemia. These results demonstrated protective effects of insulin in limiting infarction and improving contraction following MI/R when euglycaemia is maintained.

3.3 Myocardial apoptosis

To investigate how hyperglycaemia may render insulin ineffective in protecting heart from MI/R injury, additional experiments were performed to analyse apoptotic cell death, a major portion of cell loss during acute MI/R in order to determine the susceptibility of the anti-apoptotic effect of insulin to hyperglycaemia. As summarized in Figure 3, administration of insulin even at the absence of excessive plasma glucose lowering (HI/NG) significantly reduced MI/R-induced cardiomyocyte apoptosis, as evidenced by reduced caspase-3 activation and reduced TUNEL-positive staining (HI/NG vs. NI/NG, P < 0.01). However, high insulin failed to reduce cardiomyocyte apoptosis when plasma glucose remained at high levels (HI/HG vs. NI/NG, P > 0.05; HI/HG vs. HI/NG, P < 0.01), suggesting that hyperglycaemia abolished the anti-apoptotic effect of insulin in cardiomyocytes.

3.4 Coronary blood flow and vascular cell apoptosis

Previous studies by other investigators as well as our laboratory have demonstrated that insulin improves coronary perfusion, which indirectly protects cardiomyocytes from I/R injury.\textsuperscript{13,14,19} To determine whether the vasculoprotective effect of insulin was altered by hyperglycaemia, we determined the coronary blood flow and coronary vascular cell apoptosis in I/R coronary artery. No difference in coronary blood flow was observed among groups at baseline. Interestingly, insulin administration (HI/NG) remarkably increased coronary blood flow during reperfusion, which was modestly inhibited by hyperglycaemia (Figure 4A). Similarly, high insulin resulted in significantly reduced coronary vascular cell apoptosis as determined by caspase 3 activation and TUNEL staining, and this reduction was again blocked in the presence of hyperglycaemia (Figure 4B–D).

3.5 Myocardial inflammation and oxidative stress

We next sought to examine how insulin and glucose affect myocardial inflammatory response and oxidative stress, which contribute significantly

![Figure 3](image-url) Myocardial apoptosis following MI/R. (A) Representative photomicrographs of caspase-3 activation by immunohistochemistry in ischaemic cardiac tissue (400 × magnification). (B) Representative confocal images of cell apoptosis stained green by TUNEL in ischaemic cardiac tissue. Total nuclei were stained blue with DAPI, and all cardiomyocytes were stained red with anti-α-sarcomeric actin. (C) Apoptotic index expressed as the number of positively stained cells/total cells × 100%. n = 8 per group, means ± SEM. *P < 0.05, **P < 0.01.
to MI/R injury including cardiomyocyte apoptosis and endothelial dysfunction. HE staining of the paraffin sections of I/R myocardium revealed extensive leucocyte infiltration into the epicardium, subepicardium, and perivascular tissues, particularly in both HG groups (Figure 5A). Tissue MPO levels and the number of observed polymorphonuclear neutrophils both quantitatively confirmed intensified leucocyte infiltration in the high glucose-exposed I/R myocardium (Figure 5B and C). Insulin treatment led to decreased leucocyte infiltration under clamped euglycaemia, but paradoxically increased both leucocyte counts and MPO levels when hyperglycaemia prevention failed.

The MDA level and SOD activity in both necrotic and non-ischaemic tissues were determined to represent the lipid peroxidation and intrinsic antioxidative capacity of the I/R myocardium. As shown in Figure 5D and E, there was no significant difference in MDA level or SOD activity of the non-ischaemic myocardium among groups. I/R-induced MDA elevation was further intensified in hyperglycaemic groups, and significantly reduced in the HI/NG but not in the HI/HG group. Moreover, SOD activity was slightly reduced after I/R in control animals (NI/NG) and was significantly reduced in both high-glucose groups. No significant change was observed in insulin-treated groups.

3.6 Cardiomyocyte survival following hypoxia and reoxygenation in vitro

To further elucidate the molecular mechanism by which hyperglycaemia blunts insulin’s cardioprotective effects, we performed additional experiments using NRCMs. Consistent with data from animal models, trypan blue exclusion assay revealed a significant lower survival rate of NRCMs exposed to high glucose after hypoxia/reoxygenation (4 h/2 h), compared with cell cultured at either normal glucose concentration or high mannitol concentration used as hyperosmolar control (Figure 6A). More importantly, the ability of insulin to promote survival was again markedly blocked by the presence of high glucose. Cell necrosis and apoptosis were evaluated by LDH release assay and Annexin V-FITC/PI flow cytometry, respectively, and resulted in a similar inhibitory effect of high glucose on insulin action (Figure 6B and C).

3.7 O-glycosylation of IRS-1 and phosphorylation of IRS-1 and Akt

An increased glucose level is linked to up-regulated HBP with subsequently increased O-glycosylation of proteins. Cell lysates were immunoprecipitated with anti-IRS-1 antibody and immunoblotted with anti-0-GlcNAc antibody. As shown in Figure 6D, cells exposed to high glucose exhibited remarkably elevated IRS-1 glycosylation compared with those in normal glucose medium. More importantly, insulin-induced slight increase in IRS-1 glycosylation was further intensified by the presence of high glucose. As 0-GlcNAc modification is suggested to play as a dynamic negative regulator against protein phosphorylation, we next tested the hypothesis that increased IRS-1 glycosylation was accompanied by decreased tyrosine phosphorylation of IRS-1 and Akt.

As expected, insulin-stimulated tyrosine phosphorylation of IRS-1 and Akt was decreased by 45 and 40%, respectively, in the presence of...
high glucose (Figure 6E and F). These data indicated that insulin cardio-protection was affected, at least in part, by O-glycosylation of the insulin-signalling proteins in MI/R.

3.8 Inhibition of O-glycosylation under high glucose enhanced insulin protective signalling

To further evaluate the role of glycosylation in blocking the cardioprotective effect of insulin, NRCMs were pretreated with DON, a specific inhibitor of the rate-limiting enzyme (glutamine:fructose-6-P amidotransferase, GFAT) of the HBP, and revealed significant reduction in IRS-1 glycosylation as well as increases in IRS-1 and Akt phosphorylation in the presence of high glucose culture (see Supplementary material online, Figure S3D–F). More importantly, the inhibition of high glucose-induced O-GlcNAc modification by DON resulted in robust reduction in hypoxia/reoxygenation injury, as evidenced by an increased survival rate and decreased necrotic and apoptotic cell death (see Supplementary material online, Figure S3A–G).
4. Discussion

In the present study, we have provided direct evidence for the first time from a clinically relevant large animal model that hyperglycaemia aggravates MI/R injury, and blunts insulin-induced increases in post-ischaemic cardiac contractility and coronary perfusion, as well as decreases in inflammatory injury and myocardial infarction. The underlying mechanisms that hyperglycaemia attenuates insulin cardioprotection involve hyperglycaemia-induced O-GlcNAc modification and subsequent reduced phosphorylation of insulin-signalling proteins.

Hyperglycaemia is prevalent in AMI patients both upon admission and after GIK administration, and has been recognized as an independent predictor of both mortality and in-hospital morbidity with or without pre-existing diabetes mellitus. More importantly, hyperglycaemia has also been involved in recent controversies over GIK efficacy in AMI treatment. The 1995 DIGAMI Study demonstrated in post-AMI patients a 29% reduction in 1-year mortality due to GIK therapy, whereas the subsequent DIGAMI 2 and CREATE-ECLA trials failed to reconfirm the benefits of GIK treatment, and hence raised questions regarding its validity. Data from two latest clinical trials demonstrated reduced incidence of low cardiac output episodes following GIK administration after aortic valve replacement for aortic stenosis in patients with LV hypertrophy, and highlighted the significance of immediate GIK use in reducing rates of cardiac arrest and in-hospital mortality in patients with acute cardiac syndrome. In retrospect, GIK-infusion protocols differ in dosing regimens, initiation time, and duration. The lack of uniform target and fine control of the blood glucose levels in individual subjects also fuelled the divergent results. Importantly, glycaemic control has been previously reported to exclusively contribute to the survival benefit of insulin therapy in critically ill patients. In the Leuven study for example, Van den Berghe et al. has demonstrated that the dramatic mortality reduction in patients from surgical ICU following insulin therapy was due to successful glycaemic control by insulin. However, the significance of normoglycaemia maintenance in GIK therapy in MI/R is still inadequately evidenced.

Our previous studies in vivo rodent models of MI/R have shown that hyperglycaemia significantly exacerbates cardiac injury, and blunts the cardioprotective effect of GIK. This served as impetus for our hypothesis that strict glucose control by insulin may play a crucial role in GIK-mediated cardiac benefits in AMI patients, which could also be a likely explanation for the discrepancy between existing basic and clinical studies. To verify this hypothesis, we established a preclinical canine...
model of MI/R and clamped the blood glucose and insulin to either normal or high levels via blocking endogenous insulin secretion and controlled intravenous infusion of exogenous insulin and glucose. This clinically relevant large animal model thus provided a unique opportunity to demonstrate the impact of glycemic control on insulin cardioprotection in MI/R. Consistent with previous reports, data from the present study validated the detrimental effects of hyperglycaemia on post-ischaemic myocardium and demonstrated that insulin, at clamped blood glucose levels, reduced infarct size, improved contractile function, and increased coronary flow. The increased coronary flow in response to exogenous insulin may directly result from insulin-mediated vasodilation via the production of nitric oxide.13,14 Meanwhile, insulin-induced survival benefit in coronary vascular cells as observed in this study may also help preserve the endothelial-dependent vasorelaxation, hence increases coronary perfusion and accelerate functional recovery. More importantly, failure in glycemic control almost abolished the cardioprotective effects of insulin afforded under euglycaemia, including reductions in cell necrosis and apoptosis, and decreases in inflammatory and oxidative injury. These data are in line with previous animal studies from our laboratory and other’s that are suggestive of anti-apoptotic and anti-inflammatory properties of insulin via PI3-kinase–Akt–eNOS signalling in MI/R.3,8–10,18,28 These results, derived from a clinically relevant large animal model, suggest that insulin-titrated maintenance of euglycaemia is essential for the overall insulin cardioprotection against MI/R injury. Therefore, it is crucial that optimal glycemic control be achieved via frequent blood glucose monitoring and balanced insulin administration, to reap the greatest benefits of insulin therapy post-MI/R, and meanwhile avoid iatrogenic hypoglycaemia.

Several explanations have been proposed for the detrimental role of hyperglycaemia in myocardial insulin signalling. For example, the beneficial effects of insulin therapy might have been overweighed by the well-known cardiotoxicity of hyperglycaemia.29 The presence of hyperglycaemia during myocardial ischaemia is closely associated with insulin resistance, which in turn attenuates cardiac sensitivity to exogenous insulin administration.30,31 In addition, increased flux of glucose through the HBP has been hypothesized to mediate many of the adverse effects of hyperglycaemia and implicated in the development of insulin resistance. Several studies indicated that in peripheral tissues, such as skeletal muscle and fat, increased activity of the HBP, at least in part, induced insulin resistance by impairing early steps in insulin signalling, including insulin receptor activation, IRS-1 phosphorylation, and PI3-kinase activation.32 The reversible O-GlcNAc modification of proteins has been suggested by many investigators as a mechanism by which HBP mediates could cause insulin resistance. Of particular interest in the context of insulin resistance is that IRS-1 and IRS-2 and probably also GLUT 4 are subject to O-GlcNAcylation.32 The PI3-kinase/Akt signalling pathway is known to play a central role in the regulation of cell survival during I/R injury. We have previously demonstrated in rodent models of MI/R that hyperglycaemia inhibited insulin-induced PI3 K/Akt activation.27 In the present study, in cultured neonatal cardiac myocytes exposed to high glucose, we observed a significant increase in O-GlcNAc modification of IRS-1, and remarkable inhibition in early steps of insulin signalling, including tyrosine phosphorylation of the IRS-1 and impairment in the sequential activation of Akt. It should be noted that insulin also resulted in a slight increase in IRS-1 glycosylation possibly through increasing intracellular glucose flux. And there are studies showing that increased O-GlcNAc levels improved contractile function and decreased tissue injury after reperfusion.23 Thus, we speculate that there should be a threshold for protein O-GlcNAcylation to switch on the inhibition of protein phosphorylation.

One major limitation of the present study is that dogs might develop substantial and variable coronary collateral circulation, though acute occlusion/reperfusion process in our canine model of MI/R was unlikely to allow collateral compensation in such a short term.14 In addition, the 25 mM in vitro high glucose culture failed to well match the plasma high glucose levels in animal models which represented the majority of clinically reported hyperglycaemia. We chose this high glucose concentration since it is able to mimic a comparable cardiomyocyte injury as seen in vivo myocardium exposed to hyperglycaemia, and it has been well accepted and widely used in previous in vitro studies.35,36

In summary, our study in a preclinical large animal model demonstrates that hyperglycaemia blunts insulin protection against MI/R injury and reveals the role of O-GlcNAc modification in hyperglycaemia-induced impairment of insulin cardioprotection. These findings highlight the significance of hyperglycaemia prevention in GIK cardioprotection against MI/R injury and suggest inhibition of O-GlcNAc modification of insulin-signalling proteins as a potential cardioprotective strategy for hyperglycaemia-associated myocardial injury.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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Conflict of interest: none declared.

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Hyperglycaemia blunts insulin cardioprotection


